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Structure/Function Characterization of μ-Conotoxin KIIIA, an Analgesic, Nearly Irreversible Blocker of Mammalian Neuronal Sodium Channels*[S]

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Peptide neurotoxins from cone snails continue to supply compounds with therapeutic potential. Although several analgesic conotoxins have already reached human clinical trials, a continuing need exists for the discovery and development of novel non-opioid analgesics, such as subtype-selective sodium channel blockers. μ-Conotoxin KIIIA is representative of μ-conopeptides previously characterized as inhibitors of tetrodotoxin (TTX)-resistant sodium channels in amphibian dorsal root ganglion neurons. Here, we show that KIIIA has potent analgesic activity in the mouse pain model. Surprisingly, KIIIA was found to block most (>80%) of the TTX-sensitive, but only ~20% of the TTX-resistant, sodium current in mouse dorsal root ganglion neurons. KIIIA was tested on cloned mammalian channels expressed in *Xenopus* oocytes. Both $Na_V1.2$ and $Na_V1.6$ were strongly blocked; within experimental wash times of 40–60 min, block was reversed very little for $Na_V1.2$ and only partially for $Na_V1.6$. Other isoforms were blocked reversibly: $Na_V1.3$ (IC_{50} 8 μM), $Na_V1.5$ (IC_{50} 284 μM), and $Na_V1.4$ (IC_{50} 80 nM). "Alanine-walk" and related analogs were synthesized and tested against both $Na_V1.2$ and $Na_V1.4$; replacement of Trp-8 resulted in reversible block of $Na_V1.2$, whereas replacement of Lys-7, Trp-8, or Asp-11 yielded a more profound effect on the block of $Na_V1.4$ than of $Na_V1.2$. Taken together, these data suggest that KIIIA is an effective tool to study structure and function of $Na_V1.2$ and that further engineering of μ-conopeptides belonging to the KIIIA group may provide subtype-selective pharmacological compounds for mammalian neuronal sodium channels and potential therapeutics for the treatment of pain.

Venoms are a rich source of neuroactive compounds that target various ion channels and receptors with exquisite potency and selectivity (1–4). There is a continuing need for more subtype-selective pharmacological agents against sodium channels (5), and cone snail venoms provide a unique pharmacopoeia of diverse sodium channel-targeting toxins, including channel blockers as well as inhibitors of channel inactivation (6–18). μ-Conotoxins are short peptides that potently block sodium channels (Table 1). The first μ-conotoxins to be discovered from venom of *Conus* snails, GIIIA, GIIIB, GIIIC, and PIIIA, were paralytic in fish and potently inhibited skeletal muscle sodium channels in amphibian and mammalian systems.

Recently, a second group of μ-conotoxins has been identified that, in contrast to previously characterized peptides that targeted the skeletal muscle sodium channels, inhibited TTX-resistant (TTX-r)[4] sodium channels when screened on amphibian neuronal preparations (19–21). This group of conotoxins includes μ-conotoxin SmIIIA from *Conus stercusmuscarum* and μ-conotoxin KIIIA from *Conus kinoshitai* (Fig. 1). Structural and functional studies on peptides in this group to date suggest that amino acid residues in the C-terminal region of these peptides, including Trp and His (see Table 1), are important for function (19, 22).

It is widely believed that TTX-r sodium channels are potential molecular targets for analgesic therapy (23, 24). In the peripheral nerves of mammals, there are two TTX-r subtypes, $Na_v1.8$ and $Na_v1.9$; a variety of data suggest that these may participate in pain signaling. Thus, inhibition of TTX-r channels is a postulated analgesic mechanism that needs to be experimentally evaluated. Given the ability of the KIIIA group of μ-conotoxins to inhibit amphibian TTX-r sodium channels, we tested whether μ-conotoxin KIIIA is analgesic

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and supplemental Table S1.

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[4] The abbreviations used are: TTX-r, tetrodotoxin-resistant; TTX-s, TTX-sensitive; DRG, dorsal root ganglion; Na_V, voltage-gated sodium channel; KIIIA, μ-conotoxin KIIIA; mNa_V, Na_V cloned from mouse; rNa_V, Na_V cloned from rat; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.

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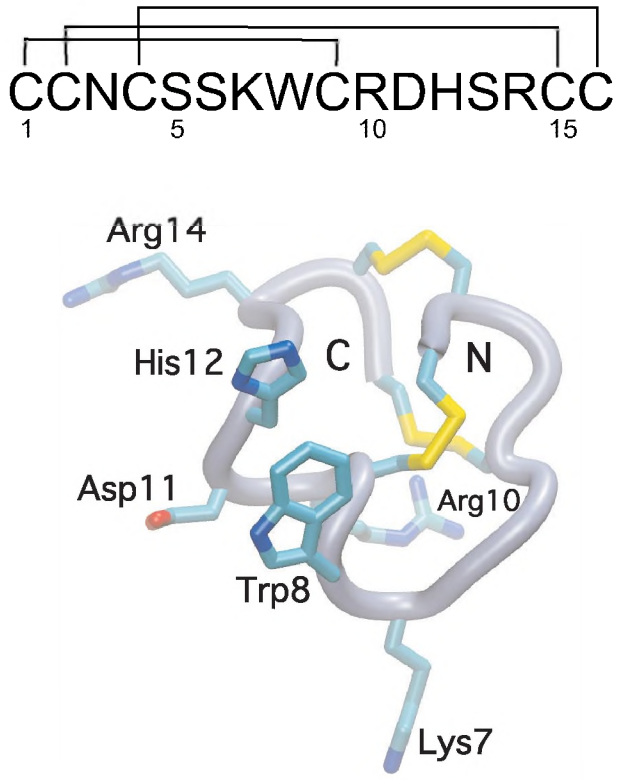

FIGURE 1. **Structure of KIIIA.** Sequence and disulfide connectivity is shown in the *upper panel*. Three-dimensional model structure was generated using molecular dynamics simulation starting with the solution structure of SmIIIA, as described (20). Amino acid residues identified in this work as important for blocking mammalian sodium channels are shown.

TABLE 1

Sequences of six μ-conotoxins belonging to the KIIIA clade

PIIIA and GIIIA are also shown for comparison. # denotes C-terminal amidation.

Conotoxin	Structure	Reference
KIIIA	CCN----CSSKWCRDHSRCC#	(20)
SIIIA	ZNCCNG--GCSSKWCRDHARCC#	(20)
SmIIIA	ZRCCNGRRGCSSRWCRDHSRCC#	(19)
MIIIA	ZGCCNVPNGCSGRWCRDHAQCC#	(21)
CnIIIA	GRCCDVPNACSGRWCRDHAQCC#	(21)
CIIIA	GRCCEGPNGCSSRWCKDHARCC#	(21)
PIIIA	ZRLCCGFOKSCRSRQCKOH-RCC#	(10)
GIIIA	RDCCTOOKKCKDRQCKOQ-RCCA#	(12)

and demonstrate that, as predicted, it has analgesic activity. However, when we evaluated the specificity of μ-conotoxin KIIIA against various mammalian sodium channel subtypes, we found that this peptide blocked only a minor fraction of the total TTX-r sodium channel currents in dorsal root ganglion (DRG) but it blocked, almost irreversibly, rat $Na_v1.2$ neuronal sodium channels. Our results validate the KIIIA group of μ-conotoxins as a rich pharmacopoeia of selective blockers of mammalian neuronal sodium channels and as potential therapeutics for treatment of pain and other neurological disorders.

EXPERIMENTAL PROCEDURES

Molecular Modeling—An initial model of KIIIA was created with the MODELLER (6v2) program using the structure of SmIIIA (Protein Data Bank accession code 1Q2J, representative structure 13) as a template, following the procedure described earlier (20, 21). Analogs were built into the initial model of KIIIA using InsightII (Accelrys, 2000). All structures were subjected to molecular dynamics simulation using the GROMACS (v3.3.1) package of programs applying a similar approach as had been used to model KIIIA previously. All simulations consisted of an initial minimization of water molecules followed by 100 ps of molecular dynamics with the peptide fixed. The restraints on the peptide were then removed and molecular dynamics continued for a further 10 ns.

Chemical Synthesis of Conotoxins—Conotoxin analogs were synthesized on solid support using standard Fmoc protocols as described previously (20). The peptides were cleaved from the resin and purified by reversed-phase HPLC. The identities of peptides were confirmed by mass spectrometry. Oxidative folding was carried out for 2 h in buffered solution (0.1 M Tris-HCl, pH 7.5) containing 1 mM EDTA, 1 mM reduced and 1 mM oxidized glutathione. The refolded peptides were purified by semi-preparative HPLC.

Inflammatory Pain Assay—Swiss Webster mice (ranging from 25 to 30 g) were placed in individual open glass cylinders for observation. Fifteen minutes prior to intraplantar injection of 10 μl of 4.4% formalin into one hind paw, mice were intraperitoneally injected with 10 or 20 nmol of test peptide or saline solution (vehicle control). Mice (two at a time) were observed for 50 min, and paw-licking time was determined every 5 min in 2-min intervals. The acute (first) phase of the nociceptive response was quantified within the first 5 min. The inflammatory (second) phase was quantified from 20–30 min. For each dose of peptide, at least four animals were tested. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Whole Cell Voltage Clamping of Mouse DRG Neurons—Adult mouse (Swiss Webster) DRG neurons were dissociated and voltage-clamped as described previously (21). Briefly, extracellular solution was (in mM): NaCl, 140; KCl, 3; $MgCl_2$, 1; $CaCl_2$, 1; HEPES, 20; pH 7.3. Recording pipettes (1–2 MΩ) contained (in mM): CsF, 140; NaCl, 10; EGTA, 1; HEPES, 10; pH 7.3. TTX-sensitive currents were recorded from large neurons whose sodium currents could be totally blocked by 1 μM TTX, and TTX-resistant currents were recorded from small neurons in the presence of 1 μM TTX. Conotoxins were dissolved in extracellular solution, and toxin exposures were conducted in static baths. The membrane potential was held at −80 mV, and sodium channels were activated by a 50-ms test pulse to 0 mV, applied every 20 s. Each test pulse was preceded by a −120 mV prepulse lasting 50 ms. Current signals were low pass-filtered at 3 kHz, digitized at a sampling frequency of 10 kHz, and leak-subtracted by a P/6 protocol using in-house software written in LabVIEW (National Instruments, Austin, TX).

Single-channel Recordings from Planar Lipid Bilayers—Membrane fractions containing voltage-gated sodium channels from rat brain were isolated using standard procedures (25, 26) and stored at −80 °C. Prior to experiments, membrane fractions were incubated with batrachotoxin (1 μM) for a minimum of 1 h. A horizontal chamber was used with two compartments (each ~200 μl) for the "extracellular" and "intracellular" solutions, separated by a plastic partition penetrated by a hole of diameter ~60–100 μm. The current offset was set to zero in

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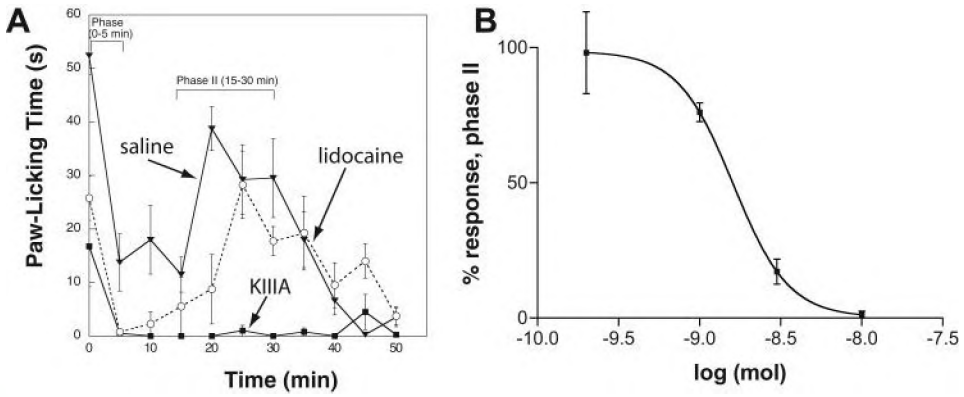

FIGURE 2. **Analgesic activity of KIIIA in the inflammatory pain assay in mice.** KIIIA was dissolved in saline and administered intraperitoneally 15 min prior to the injection of formalin into a paw as described under "Experimental Procedures." *A*, time course of a paw-licking time following injection of saline (*black triangles*), 20 mg/kg dose of lidocaine (*open circles*), or 10 nmol (equivalent of 0.7 mg/kg) of KIIIA (*black squares*). Phase I response is defined as the cumulative licking time between 0 and 5 min following formalin injection, whereas phase II response (reflecting activity in response to inflammatory pain) is defined as the cumulative licking time between 15 and 30 min. Each dose was tested in at least four animals. *B*, dose-response relationship of the analgesic activity of KIIIA during phase II. Data were normalized to the response to saline solution alone. ED_{50} of 1.6 nmol/mouse (0.1 mg/kg) was calculated using Prism software.

symmetric solutions before forming the bilayer. The bilayer was formed by painting a solution of lipid across the hole (50 mg of total lipid/ml in decane, POPE:POPC, 4:1). Lipids were from Avanti Polar Lipids. Records were made at room temperature using symmetrical solutions of (concentrations in mM) NaCl, 200; MOPS, 10; and EDTA, 0.1; pH 7.0. Aliquots of membrane fractions (~1 μl) were pipetted directly over the bilayer, and incorporation of a single channel was detected as an increase in current amplitude of ~1 pA at ± 60 mV. Channel orientation was deduced from the increased frequency of closures at −70 mV (extracellular side defined as 0 mV). Toxin was added to the extracellular side at 500 nM by perfusing the appropriate chamber with toxin-containing solution (2 × 400 μl), and toxin-blocking events were observed as step decreases in single-channel amplitude. Data were collected and analyzed using pClamp 9.2 software, an Axopatch 200 amplifier, a Digidata 1322A acquisition system (Molecular Devices Corp., Sunnyvale, CA), and SigmaPlot 8.0 (Systat Software, Inc., San Jose, CA).

Generation of Na_V Clones and RNA Preparation—The cDNA clones were kindly provided by Drs. Alan Goldin and Gail Mandel. Approximately 20 ng of cDNA for each Na_v channel isoform was transfected into electrocompetent cells and plated on an agar plate with the antibiotic. Ten clones were picked and grown overnight in 5 ml of Luria Bertani medium. Of the 10 inoculated media, the cDNA was isolated from five and screened by BamHI digestion. One clone from each that yielded the correct fragments was grown in 50 ml of Luria Bertani medium with the antibiotic to obtain enough cDNA for sequencing. The correct cDNA for each isoform was linearized with NotI and transcribed with T7 RNA polymerase using the mMessage mMachine RNA transcription kit (Ambion, Austin, TX) for generation of capped cRNA. The cRNA was purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA). The concentration of the cRNA was determined by UV spectroscopy at 260 nm using a Milton Roy Spectronic 1001.

Harvesting, cRNA Injection, and Voltage Clamping of Xenopus Oocytes—Oocytes were harvested, prepared, and injected with cRNA as previously described (27). A given oocyte was injected with 30 nl of cRNA for one of the following sodium channel isoforms: $rNa_V1.2$, $rNa_V1.3$, $rNa_V1.4$, $rNa_V1.5$, $mNa_V1.6$, or $rNa_V1.7$ (1.5, 15, 0.6, 3, 30, or 15 ng, respectively). Oocytes were two-electrode voltage-clamped using microelectrodes containing 3 M KCl (<0.5 MΩ). Sodium currents were acquired using a holding potential of −80 mV and stepping to a value between −20 and 0 mV (depending on Na_V subtype) for 50 ms every 20 s and processed as described above for clamping of neurons. Toxin exposures were in static baths to conserve material. Off-rate constants were determined from single-exponential fits of the time course of recovery from block following peptide washout, and the on-rate constants were obtained from the slopes of k_{obs} *versus* [peptide] as done previously (19). Expression and electrophysiological measurements of $rNa_v1.8$ channels were performed as described in Ref. 15. All electrophysiological recordings were conducted at room temperature (~20 °C).

RESULTS

KIIIA Is a Potent Analgesic Compound—Because many sodium channel blockers possess analgesic activity, and KIIIA was found to block TTX-r sodium channels in amphibian preparations (20), we first tested this peptide for analgesic activity following systemic delivery in the formalin-induced pain assay in mice. As shown in Fig. 2, intraperitoneal injection of KIIIA significantly reduced paw licking during both phase I and phase II. Compared with lidocaine (20 mg/kg^{-1} intraperitoneal, ~3 times the normal maximum systemic dose for adult humans), KIIIA appeared to be a more effective analgesic, in particular during phase II, which reflects inflammatory pain. This analgesic effect was dose-dependent (Fig. 2*B*), with an ED_{50} of 1.6 nmol/mouse (equivalent of 0.1 mg/kg). Using a rotarod test, no motor impairment was observed at a dose of 3 nmol, indicating a separation of the toxic and analgesic activity of the peptide; some motor impairment, but no apparent paralytic activity, was found at the highest dose of KIIIA tested, 10 nmol.

Unexpected Specificity of KIIIA for Cloned Rat and Mouse Sodium Channels—We examined the ability of KIIIA to block sodium channels in mouse DRG neurons. As shown in Fig. 3, KIIIA (5 μM) largely blocked (>80%) TTX-sensitive (TTX-s) but only partially blocked (~20%) TTX-r sodium channels. Increasing the concentration of KIIIA to 50 μM did not yield any additional block of the TTX-r sodium current in DRG neurons. Consistent with the above observations, only TTX-s A-compound action potentials were abolished by 5 μM KIIIA, whereas

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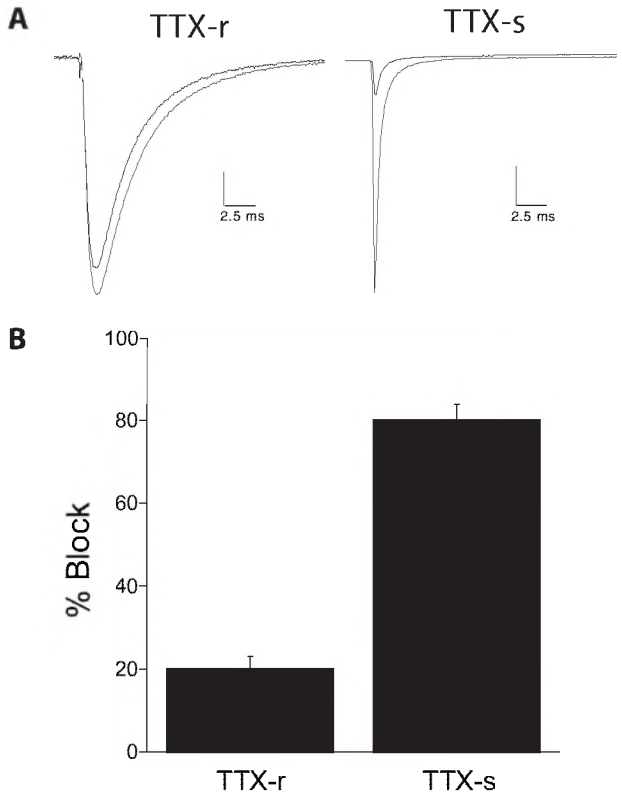

FIGURE 3. **KIIIA blocks mouse TTX-sensitive sodium channels in DRG neurons.** *A*, effects of KIIIA on sodium currents in dissociated DRG neurons. *Left*, representative TTX-resistant sodium currents recorded before (control trace, *gray*) and following ~20 min of exposure to 5 μM KIIIA (*black trace*). *Right*, representative TTX-sensitive sodium currents recorded before (control trace, *gray*) and following ~20 min of exposure to 5 μM KIIIA (*black trace*). Each trace represents the average of five responses. *B*, percent of block of the peak of TTX-resistant and TTX-sensitive sodium currents following ~20 min of exposure to 5 μM KIIIA. Mean and S.D. values were calculated from $n = 4$ to 5 using Excel software.

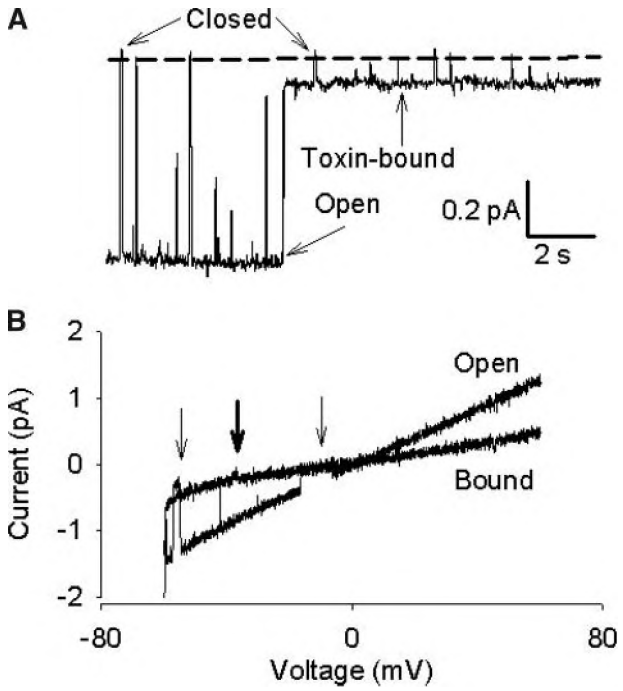

FIGURE 4. **KIIIA blocks sodium channels from rat brain membrane preparations.** *A*, steady state single-channel record from a rat brain channel at −40 mV, showing a toxin binding event. *B*, single-channel current-voltage relations obtained during voltage ramps (−60 to +60 mV in 3.5 s), for the unbound (*open*) channel and for the KIIIA-bound channel. Brief, intrinsic gating closures are seen from either open (*bold arrow*) or toxin-bound states (*light arrows*), indicating a small residual current through the single channel when KIIIA is bound.

C-compound action potentials were slowed and only slightly attenuated by the toxin. C-compound action potentials that persist in the presence of TTX were unaffected by KIIIA (data not shown).

KIIIA also caused discrete block of unitary currents from Na_V channels incorporated into lipid bilayers from rat brain membranes (Fig. 4). These records showed no dissociation (unblocking) transitions in 63 min of recorded blocked time from four experiments, placing a lower limit on the mean blocked time of 15.8 ± 5.8 min (mean ± S.E.). Thus, block is effectively irreversible on the experimental time scale. The small residual unblocked current, seen in Fig. 4 for the KIIIA-bound channel, parallels single-channel and whole cell data for some derivatives of μ-GIIIA (28) and the residual TTX-s current at high KIIIA concentrations in Figs. 3*A* and 5*A*.

To characterize the activity of KIIIA on mammalian sodium channels in further detail, various cloned α-subunits of rat or mouse sodium channels were expressed in oocytes. Fig. 5*A* illustrates representative traces of the block of $rNa_V1.2$ and 1.4 by KIIIA, and Table 2 summarizes the results with seven subtypes of sodium channels. All five neuronal subtypes tested, $rNa_v1.1$, $rNa_v1.2$, $rNa_v1.3$, $mNa_v1.6$, and $rNa_v1.7$, were blocked by KIIIA: $rNa_v1.2$ was essentially irreversibly blocked with a k_{on} of 0.3 $\mu M^{-1} \cdot min^{-1}$. In contrast to $rNa_v1.2$, $rNa_v1.3$ was reversibly inhibited with an IC_{50} of 8 μM. Inhibition of $rNa_v1.1$ and $rNa_v1.7$ was characterized by very slow on- and off-rates. The skeletal muscle subtype $rNa_v1.4$ was reversibly blocked with a relatively fast on-rate and a calculated K_D of 0.05 μM, close to the IC_{50} of 0.08 μM. Interestingly, KIIIA was significantly less potent against heart muscle subtype $rNa_v1.5$ (estimated IC_{50} 284 μM). Application of KIIIA at 5 μM did not affect $rNa_v1.8$ co-expressed with β1 subunits in oocytes (data not shown).

Structural Determinants of KIIIA Block—To identify amino acid residues in KIIIA responsible for its activity against neuronal and skeletal muscle subtypes, we designed and chemically synthesized "Ala-walk" and related analogs of KIIIA (Table 3 and supplemental Table S1). Two analogs of KIIIA contained additional two-amino acid extensions at the N terminus, ZN- or ZR- (where Z is pyroglutamate), as found in the related μ-conotoxins SIIIA and SmIIIA, respectively (Table 1). All analogs were synthesized using Fmoc chemistry and subjected to the oxidative folding procedure used previously to produce KIIIA (20). When the folding was performed in the presence of oxidized and reduced glutathione, a number of the analogs yielded one major oxidation product, but several analogs produced two folded species in approximately equal quantities (supplemental Fig. S1). To assess which of the two products was more likely to be the correctly folded form with the native connectivity of disulfide bridges, we compared functional and structural properties of both products. The individual folding species were tested for their ability to block $rNa_v1.2$ sodium

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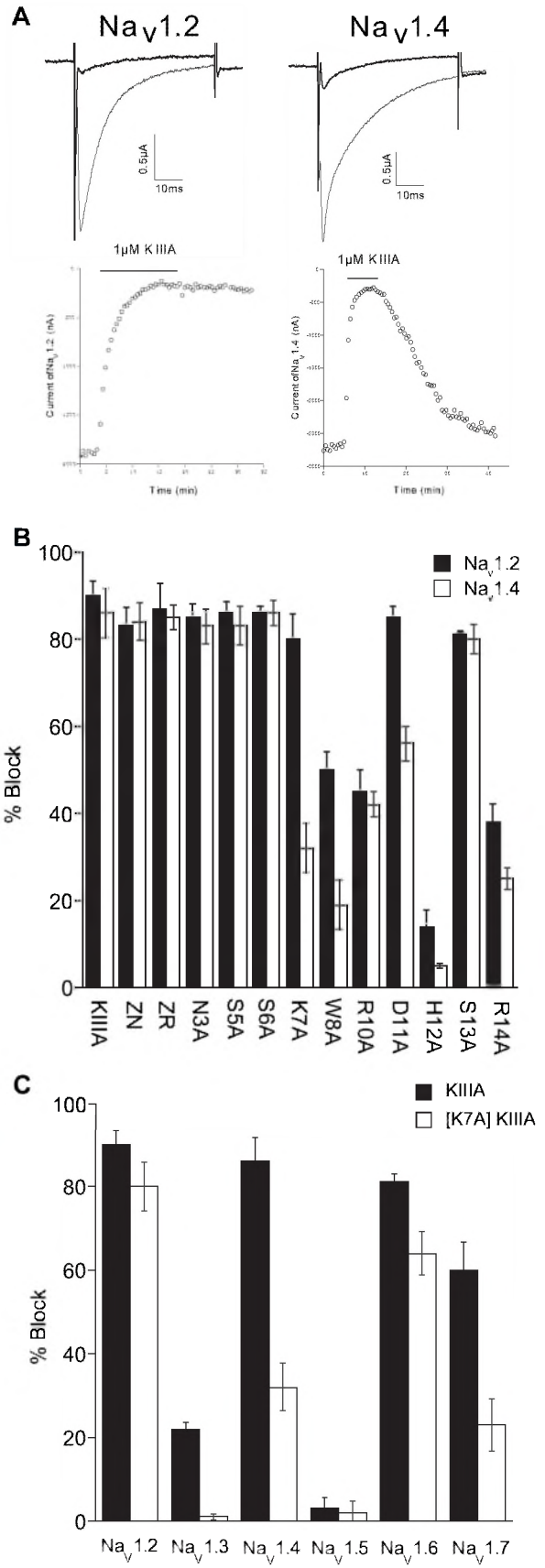

FIGURE 5. **Block by KIIIA and its analogs of rNa$_V$1.2 and 1.4 expressed in *Xenopus* oocytes.** *A*, representative traces from voltage-clamp recordings were performed as described under "Experimental Procedures." KIIIA and the analogs were each tested at a concentration of 1 μM on oocytes expressing rNa$_V$1.2 or rNa$_V$1.4. In each panel, the *top row* shows superimposed recordings before (control, *gray trace*) and during (*black trace*) exposure to indicated peptide. The *bottom row* shows the time course of block and recovery, where the *black bar* above each plot indicates when the peptide was present. Block by KIIIA of rNa$_V$1.2 was irreversible but that of rNa$_V$1.4 was reversible. *B*, comparison of the activity of KIIIA and its analogs against rNa$_V$1.2 and 1.4. Modification of residues in the N-terminal portion of the peptide had minimal effects in contrast to those in the C-terminal portion. Remarkably, the K7A substitution attenuated the activity against rNa$_V$1.4 but not rNa$_V$1.2. Mean and S.D. values were calculated from at least three independent experiments using Excel software. *C*, comparison of the block of six Na$_V$s by KIIIA and K7A. All Na$_V$s were from rat, except Na$_V$1.6, which was from mouse.

TABLE 2

Inhibition by KIIIA of cloned sodium channels expressed in *Xenopus* oocytes

Rate constants were determined as described under "Experimental Procedures." Standard deviation and 95% Confidence Intervals (95% C.I.) were calculated from at least three independent experiments using Prism software. *n.a.*, not available because slow kinetics precluded steady state from being achieved within the experimental time frame.

Sodium channel	k_{on}	k_{off}	K_d	IC_{50} (95% C.I.)
	$\mu M^{-1}.min^{-1}$	min^{-1}	μM	μM
rNa$_V$1.1	0.042 ± 0.007	0.012 ± 0.004	0.29	*n.a.*
rNa$_V$1.2	0.30 ± 0.03	Irreversible[a]	0.003	*n.a.*
rNa$_V$1.3	0.08 ± 0.02	0.37 ± 0.08	4.6	8.0 (6.7–9.4)
rNa$_V$1.4	0.97 ± 0.09	0.047 ± 0.015	0.05	0.09 (0.07–0.11)
rNa$_V$1.5	-[b]	-[b]		287 (240–343)[c]
mNa$_V$1.6	0.12 ± 0.01	Partial[d]		*n.a.*
rNa$_V$1.7	0.024 ± 0.003	0.007 ± 0.002[e]	0.29	*n.a.*

[a] Although the block was essentially irreversible within the experimental time frame, the k_{off} was estimated as 0.0008 ± 0.0003 min^{-1} based on residual recovery after 20 min of wash and assuming exponential decay.
[b] Unable to be determined because block was too small and apparent kinetics too fast to be accurately measured.
[c] Extrapolated value assuming 100% block at saturating peptide concentration.
[d] Block only partially reversed following peptide washout (~40 min).
[e] k_{off} was estimated from recovery after 20 min of wash assuming exponential decay.

channels expressed in oocytes and also analyzed by ^{1}H NMR (supplemental Figs. S2 and S3). For each analog, a single product was significantly more active than others present, suggesting that it was likely to be the correctly folded form. The correct folding of these analogs was confirmed by one-dimensional ^{1}H NMR. Molecular dynamics simulations suggested that the introduced replacements (such as W8A) should not affect the conformation of the peptide and that selected misfolded forms (containing non-native disulfide bridges) should differ in overall conformation from the native one (data not shown). For the analyses of structure-activity relationships, the most active folding product of each mutant was used in subsequent experiments.

The analogs were screened at a concentration of 1 μM for their ability to block rNa$_V$1.2 and 1.4 sodium channels expressed in oocytes. The activities of the folding products of the analogs against rNa$_V$1.2 and 1.4 are compared in Fig. 5*B*, and detailed kinetic parameters of the inhibition are summarized in Table 3. Individual Ala substitution of four residues, namely Trp-8, Arg-10, His-12, and Arg-14, reduced the activity of KIIIA against rNa$_V$1.2. However, all derivatives, with the exception of W8A, showed little reversibility over the time course of the experiment. The on-rate was significantly lowered by replacement of the positively charged residues Lys-7, Arg-10, or Arg-14, whereas replacement of the

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TABLE 3

Inhibition of rNa$_V$1.2 and rNa$_V$1.4 by KIIIA and its analogs

Standard deviation was calculated from at least three independent experiments using Excel software. Peptide concentration was 1 μM for all, and inhibition (% block) was determined following 20 min of exposure to the indicated peptide. *, level of block too low to obtain reliable data.

Conotoxin[a]	rNa$_V$1.2				rNa$_V$1.4				
	Block	k_{obs}	Δk_{obs}[b]	k_{off}	Block	k_{obs}	Δk_{obs}	k_{off}	Δk_{off}
	%	min^{-1}		min^{-1}	%	min^{-1}		min^{-1}	
KIIIA	90 ± 3.3	0.36 ± 0.06	1.0	Irreversible	86 ± 5.6	0.91 ± 0.17	1.0	0.05 ± 0.017	1.0
[ZN]$_{II}$	83 ± 4.4	0.31 ± 0.17	0.9	Irreversible	84 ± 4.2	1.31 ± 0.37	1.4	0.09 ± 0.037	1.8
[ZR]$_{II}$	87 ± 5.8	0.56 ± 0.13	1.6	Irreversible	85 ± 2.8	1.69 ± 0.50	1.9	0.05 ± 0.006	1.0
[N3A]	85 ± 3.1	0.34 ± 0.08	0.9	Irreversible	83 ± 4.1	0.87 ± 0.16	1.0	0.05 ± 0.017	1.0
[S5A]	86 ± 2.7	0.41 ± 0.13	1.1	Irreversible	83 ± 4.5	0.80 ± 0.09	0.9	0.04 ± 0.003	0.8
[S6A]	86 ± 1.5	0.29 ± 0.05	0.8	Irreversible	86 ± 2.9	0.91 ± 0.15	1.0	0.04 ± 0.016	0.8
[K7A]$_I$	80 ± 5.9	0.17 ± 0.06	0.5	0.02 ± 0.007	32 ± 5.7	0.65 ± 0.14	0.7	0.37 ± 0.077	7.4
[K7Nleu]$_I$	83 ± 2.1	0.18 ± 0.04	0.5	Irreversible	33 ± 4.6	0.89 ± 0.12	1.0	0.20 ± 0.046	4.0
[W8A]$_{II}$	50 ± 4.1	0.98 ± 0.14	2.7	0.26 ± 0.075	19 ± 5.7	0.91 ± 0.16	1.0	0.86 ± 0.120	17.2
[W8L]$_{II}$	31 ± 6.6	0.91 ± 0.17	2.5	0.45 ± 0.186	15 ± 4.2	1.95 ± 0.64	2.1	1.75 ± 0.21	35.0
[$_D$W8]$_I$	5 ± 5.0	*		*	5 ± 1.0	*		*	
[R10A]$_I$	45 ± 5.0	0.13 ± 0.02	0.4	Irreversible	42 ± 2.9	0.84 ± 0.19	0.9	0.29 ± 0.140	5.8
[D11A]	85 ± 2.5	0.86 ± 0.31	2.4	Irreversible	56 ± 4.0	0.78 ± 0.13	0.9	0.21 ± 0.095	4.2
[H12A]$_{II}$	14 ± 3.8	*		*	5 ± 0.6	*		*	
[S13A]$_{II}$	81 ± 1.0	0.31 ± 0.08	0.9	Irreversible	80 ± 3.4	1.12 ± 0.59	1.2	0.08 ± 0.023	1.6
[R14A]	38 ± 7.5	0.11 ± 0.02	0.3	Irreversible	25 ± 4.4	1.12 ± 0.55	1.2	0.8 ± 0.248	16.0

[a] Subscript I or II refers to HPLC peak in supplemental Fig. S1.
[b] Normalized to the value for KIIIA.

negatively charged Asp-11 resulted in an increased on-rate (Table 3). The inhibition of rNa$_V$1.4 was reduced by the individual replacements of either Lys-7, Trp-8, Arg-10, Asp-11, His-12, or Arg-14. As for rNa$_V$1.2, the kinetics of block of rNa$_V$1.4 was influenced by substitutions of the charged residues. The replacements K7A, W8A, or D11A had more effect on the block of rNa$_V$1.4 than of rNa$_V$1.2 (Fig. 5*B*). Addition of N-terminal sequences ZN- and ZR- (as seen in SIIIA or SmIIIA, Table 1) and individual substitutions of residues 3, 5, and 6 by Ala had minimal, if any, effects.

The observation that KIIIA[K7A] and a few other analogs discriminated between rNa$_V$1.2 and 1.4 suggests that these positions might be useful for engineering subtype selectivity in μ-conotoxins. To test this, we measured the activity of KIIIA[K7A] against additional subtypes of sodium channels. As shown in Fig. 5*C*, the K7A substitution retained activity against rNa$_V$1.2, but the inhibition of rNa$_V$1.4 and 1.7 by this analog was substantially reduced compared with KIIIA.

DISCUSSION

In a quest to discover and develop novel non-opioid analgesics we have characterized the activity of KIIIA, a representative member of a newly discovered group of μ-conotoxins from fish-hunting cone snails. Our earlier work indicated that KIIIA and closely related peptides such as SIIIA potently blocked TTX-r sodium channels in amphibian neuronal preparations (19–21). Three major findings in the present investigation are that 1) in mammalian systems, KIIIA almost irreversibly blocks Na$_V$1.2, 2) replacement of specific amino acids had different effects on the block of rNa$_V$1.2 and rNa$_V$1.4, and 3) KIIIA has a potent analgesic activity following its systemic administration in mice.

This work validates the KIIIA group of μ-conotoxins as potential therapeutics for treatment of pain. We show that KIIIA is a potent, systemically active analgesic compound acting by blocking sodium channels. KIIIA produced a more potent analgesia (ED_{50} 0.1 mg/kg) than SIIIA (ED_{50} 0.9 mg/kg) (29). These findings are not surprising given that many sodium channel blockers, including TTX itself, are active in analgesia assays (30–33). Previously reported analgesic activity of subcutaneously applied μO-conotoxin MrVIB was presumed to be mediated by preferentially blocking TTX-r currents, including Na$_V$1.8 (14, 15, 34). Although we did not identify the particular subtype of sodium channels by which KIIIA mediated analgesic effect (these experiments are beyond the scope of this report), it is likely that blocking one or more peripheral neuronal subtypes of sodium channels underlies the analgesic activity. For example, Na$_V$1.7, found in peripheral sensory neurons, has been shown to play a role in communicating inflammatory pain (23, 35), and mutations in the human Na$_V$1.7 can result in the inability to experience pain (36).

A striking feature of KIIIA is its essentially irreversible block of rNa$_V$1.2. Based on residual recovery after 20 min of wash, and assuming exponential decay, we estimated k_{off} as 0.0008 ± 0.0003 min^{-1}. This finding was also supported by single-channel recordings from rat brain channels (Fig. 4). Remarkably, this near irreversibility was retained by 12 of the 14 KIIIA analogs that remained active in blocking these two channel subtypes (Table 3). The selectivity profile for KIIIA against mammalian sodium channel subtypes was found to differ significantly from those previously described for PIIIA and GIIIA. KIIIA is a nearly irreversible blocker of Na$_V$1.2, whereas PIIIA and GIIIA have IC_{50}s in the low (PIIIA) or high (GIIIA) μM range for this subtype (10, 37). It is tempting to speculate that this difference might be accounted for in part by the presence of the highly conserved Trp-8 in members of the KIIIA group of μ-conotoxins (Table 1), replacement of which resulted in lower activity and reversible block of Na$_V$1.2. Secondly, the skeletal muscle subtype was blocked more by GIIIA/PIIIA than KIIIA. Thus, there is a stark contrast in the specificity of KIIIA compared with GIIIA/PIIIA in blocking neuronal *versus* skeletal muscle subtypes. Furthermore, our structure-activity relationship data identified three positions, Lys-7, Trp-8, and Asp-11, that through replacement, either singly or in com-

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bination, may result in more selective blockers. The present structure/function data on KIIIA support its use in the study of neuronal sodium channel structure in the same way that μ-conotoxin GIIIA has contributed to defining the structure of the skeletal muscle sodium channels (38–41). Moreover, our structure-activity relationship information is critical for further discovery and development of subtype-selective sodium channel blockers via exogenomics and/or a rational design approach (42).

A puzzling feature of KIIIA activity is that it blocked only 20% of the TTX-r currents in mouse DRG neurons (Fig. 3, *A* and *B*). It is unlikely that this result is due to a differential block of $Na_V1.8$ *versus* $Na_V1.9$ in these cells because under our recording conditions the TTX-r current is essentially only due to $Na_V1.8$. Furthermore, kinetics of the control and partially blocked currents are essentially identical (see Fig. 3*A*), which is inconsistent with a differential block of these two TTX-r channel subtypes since they have very different rates of fast inactivation (43). One possible basis for the residual current at high [KIIIA] could be incomplete block of the unitary current for the channel type(s) underlying the TTX-r current, as seen for rat brain channels in Fig. 4. However, a more detailed biophysical study will be required to resolve this issue conclusively.

In summary, this work shows for the first time that μ-conotoxins can block neuronal subtypes of mammalian sodium channels, with bound times of unprecedented duration, and validate this group of peptides as potential therapeutics for treatment of pain. Results from our structure/function analysis provide strong incentives for further engineering of the KIIIA group of μ-conotoxins to obtain specific blockers of selected neuronal sodium channel subtypes that potentially might be valuable new tools for the treatment of pain (44).

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